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Effects of a Chronic 0.1 µM Arsenic Exposure on P19 Mouse Embryonic Stem Cell Differentiation and Pluripotency

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EFFECTS OF A CHRONIC 0.1 μM ARSENIC EXPOSURE ON P19 MOUSE EMBRYONIC STEM CELL DIFFERENTIATION AND PLURIPOTENCY

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Abstract

Globally, at least 140 million people are exposed to levels of arsenic greater than the World Health Organization recommended standard of 10 μg/L (10 ppb) through their drinking water and food. Epidemiological studies have correlated arsenic exposure during embryonic and fetal development with reduced birth weight, as well as muscular and neurodevelopmental deficits. The goal of the current study was to determine the mechanisms by which a chronic, low-level arsenic exposure impairs cellular development and differentiation. This was achieved by exposing P19 mouse embryonic stem cells (mESC's) to 0.1 μM arsenic (7.5 ppb), a concentration lower than the current drinking water standard, continuously for 32 weeks.

The P19 stem cell line is commonly used in developmental studies as it can be induced to form cells of all three germ layers. During the current study, a subset of cells were induced to differentiate every 4 weeks of the 32-week arsenic exposure, to assess whether arsenic impaired their ability to form mature cell lineages. Differentiating cells were collected after 5 and 9 days to examine transcript and protein expression. Previous studies of P19 cells exposed to higher arsenic concentrations (0.5 – 1 μM) reported robust differences in cell morphology, as well as reductions of transcription factors involved in neuronal differentiation, and increases in transcription factors crucial for maintaining pluripotency.

In this study of 0.1 μM arsenic, only minor and inconsistent changes in morphology were observed to result from exposure. However, arsenic induced consistent overexpression of the pluripotency markers Oct4 and Sox2 by 1.4- to 1.8-fold beginning

at week 8, while Sox2 protein expression was increased by 1.4 fold starting at week 16. Elevated expression of pluripotency-associated transcription factors is commonly observed in ESC's, but also in cancer stem cells (CSC's). Another feature of CSC's are protein expression patterns consistent with epithelial-mesenchymal transitions (EMT). A prevailing hallmark of EMT is increased expression of the cell-to-cell adhesion molecule N-cadherin, which is known to be induced by Sox2. Beginning at week 20, N-cadherin levels remained significantly increased by 1.3- to 1.9 fold through the 32-week exposure. Interestingly, as markers of pluripotency and EMT increased, the expression of the doublecortin (DCX) gene, a microtubule-associated protein of immature neurons, was consistently reduced throughout the exposure with significance of up to 2-fold decreases shown at weeks 8 and 20. However, transcript levels of Nrf2 and Hmox1, markers of oxidative stress, and MyoD, a marker of myogenesis, appeared to be unaltered throughout the exposure.

In conclusion, this study may have public health ramifications as it was shown arsenic exposure at a level lower than the current drinking water standard maintained pluripotency of ESC's and reduced neural differentiation. An EMT-like mechanism may be used to impair cellular differentiation as demonstrated by increased N-cadherin expression. Intriguingly, the observed lack of oxidative stress leaves the possibility of another mechanism, such as epigenetics, to be responsible for this reduced differentiation.

Dedication

This thesis is dedicated to my parents who have always supported my dreams and aspirations. Attending their college graduations and observing their perseverance and hard-work has served as the inspiration and motivation to pursue my own goals. I also dedicate this to my old and new friends alike. They have been there to celebrate the successes, and put things in perspective on tough days. Finally, this thesis is dedicated to my undergraduate professors and mentors at Florida State University who sparked my interest in scientific research and have continued to support my future endeavors.

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Table of Contents

List of Tables

List of Figures

Figure Page

Chapter One

Literature Review

Purpose of the Study

The main objective of this study was to determine how a chronic, low-level arsenic exposure (0.1 μM) for 32 weeks impairs cellular differentiation early in development, using P19 mouse embryonic stem cells as the model.

Routes of Arsenic Exposure

For centuries, arsenic (As) has been feared due to its harmful effects in humans, and has often been referred to as the "king of poisons" for its use in many purposeful poisonings (Hughes 2011). In recent times, human arsenic exposure is often through ingestion of food and water but also through inhalation of emissions from smelting operations and the manufacture of insecticides, pesticides, and wood preservatives (ATSDR 2014). Human arsenic exposure has also been due to mining, as arsenic is commonly found within metalliferous deposits of iron, manganese, and other metals of commercial interest. These are processed, removing arsenic from the ore of interest and leaving behind arsenic residues in mines (Camm 2004, Mandal 2002). Inorganic arsenic is used in manufacturing, commonly for semiconductor production (Park 2010). Furthermore, arsenic-based pesticides, predominantly lead arsenate, copper arsenate, and calcium arsenate, have been used across the world to reduce insect damage to crops and therefore boost agriculture production (Li 2016). A study of New Jersey farms concluded

that between the years 1900-1980 the equivalent of 15 million lbs of arsenic was applied to crops (Murphy 1998). As of 1986, arsenic-based pesticides were banned in the United States (EPA 1986).

Since arsenic is found naturally in the Earth's crust bound to metal ores such as iron and manganese, it can enter groundwater as a result of two processes: 1) either a rise in the pH of groundwater, leading to desorption of arsenic from mineral oxides such as iron or manganese, or 2) as a result of the development of a strongly reducing environment causing the desorption of arsenic from these mineral oxides and thus arsenic release into the groundwater (Smedley 2002). A number of countries have issues with arsenic in groundwater, including China, Mexico, Thailand, Argentina, and the United States, with the highest incidences of exposure seen in Bangladesh and India (Dwivedi 2015) (Table 1). For example, a 1996 study demonstrated that 50% of 400 samples of groundwater from tube wells in Bangladesh showed concentrations of arsenic greater than 50 μg/L (higher than the WHO recommendation of 10 μg/L (WHO 2015)), while a 1997 study of the tube wells of 200 villages showed that 62% had concentrations higher than 100 μg/L (Smith 2000, Quamruzzaman 1998). Arsenic contamination of groundwater in the United States is also prevalent, with an estimated 2.1 million citizens obtaining drinking water from wells with arsenic concentrations over 10 μg/L (Ayotte 2017). Naturally occurring arsenic exists in two oxidation states, either As(III) or As (V). In the U.S., As(III) is the dominant species in the Midwest, while both species are observed throughout the rest of the country (Sorg 2014).

Table 1.1. Concentrations of arsenic in drinking water from various regions around the world.

Country	Arsenic Concentration in Drinking Water	Sources
China	Sometimes >500 µg/L	He and Charlet, 2013
Northern Mexico	Up to 600 µg/L	Fisher, et al., 2017
Central Thailand	Up to 100 µg/L	Kohnhorst, et al., 2002
Central Argentina	Up to 300 µg/L	Paoloni, et al., 2009
United States (Private Wells)	Sometimes >300 µg/L	Neilson, et al., 2010
Bangladesh	Sometimes >1000 µg/L	Flanagan, et al., 2012
India (Bihar District)	Sometimes > 1500 µg/L	Chowdhury, et al., 1999; Kumar, et al., 2016

Arsenic Ingestion

In addition to the risk from drinking water, irrigation of crops can lead to arsenic uptake into various vegetables, grains, and fruits. For the most part, arsenic exposure through food is a result of high consumption levels, and not high concentration levels of the foods themselves (Gundert-Remy 2015). Exceptions to this include rice and apples, which both readily uptake arsenic. Rice is one of the most common food sources worldwide with production and consumption increasing since the 1960's (FAO 2002). A study from 2008 compared 204 rice grain samples from around the world and concluded a "normal" range of arsenic is 0.08-0.20 mg/kg. This same study found that mean arsenic concentrations in rice grown in the U.S. and Europe were both 0.198 mg/kg, which was significantly higher than rice grown in Asia (excluding rice grain from Bangladesh) with a mean arsenic concentration of 0.07 mg/kg (Zavala 2008). Furthermore, comparing two large data sets of arsenic levels in Bangladeshi rice, researchers showed that contaminated irrigation water increased the arsenic concentration of rice by 0.05 to 0.13 mg/kg, or 25-45% when compared to rice grown in uncontaminated environments (Zavala 2008). Apples

are another concern (Creger 1992). Many orchard soils have high levels of arsenic due to the previous treatment of apple trees with arsenic-based pesticides, which remain in the soil for many years after their use (Yokel 2003, Morgan 2009). From 2005 through 2011, the U.S. FDA monitored the amounts of arsenic found in apple juice from countries around the world bound for U.S. markets. These studies found total arsenic concentrations ranging from non-detectable amounts up to 45 ppb, and inorganic arsenic concentrations (the arsenic species more harmful to human health) up to 18 ppb (FDA 2011). In 2013, the U.S. FDA set the standard for arsenic in apple juice to the equivalent standard of arsenic in drinking water at 10 ppb (FDA 2013).

Developmental Arsenic Exposure

Although exposure to arsenic at any age can cause negative effects, embryonic exposure can cause deficits that may last a lifetime. Arsenic is able to pass through the blood placental barrier and accumulate in the brain, causing toxicity during embryonic development (Concha 1998, Jin 2006). A cohort study of the birth size of babies in Bangladesh in 2002-2003 concluded that low levels of arsenic exposure (less than 100 μg/L in urine) significantly reduced birth weight (Rahman 2009). Another study of 272 children from Maine in grades 3-5 showed that children drinking water with concentrations of arsenic higher than 5 μg/L scored lower on Full-Scale IQ tests and most Index scores (including perceptual reasoning, working memory, and verbal comprehension) compared to children ingesting water with lower levels of arsenic (Wasserman 2014). Furthermore, exposure to arsenic during development increases the incidences of neonatal mortality and

stillbirths, reduces birth weight, alters locomotor function, increases the incidence of neurological diseases and mental retardation, and lowers intelligence quotient scores (Hong 2012, Dakeishi 2006, Tsai 2003, Raqib 2009, von Ehrenstein 2006, Xi 2010).

Arsenic and Epidemiological Outcomes

Human exposure to arsenic causes a number of effects, most notably cancers of the skin, bladder, kidney, lungs, and liver. A 2015 review study concluded that arsenic-exposed populations showed a dose-dependent increase in skin lesions as well as basal and squamous cell carcinomas. These trends were common in Asian countries where populations were more likely to be exposed to drinking water with arsenic concentrations higher than 10 μg/L (Karagas 2015). In 2014, another cohort review study concluded that exposure to 10 μg/L arsenic in drinking water increased the risk of bladder cancer by 40%, and the mortality rate of both bladder and kidney cancer by 30%, in populations exposed to 150 μg/L arsenic in drinking water (Saint Jacques 2014). A meta-analyses conducted in 2014 of 12 studies concluded that long-term inorganic arsenic exposure through drinking water increased liver cancer mortality by nearly two-fold (Wang 2014). Similar carcinogenic findings have been observed in rodents (Tokar, et al., 2010). A study of orally administered inorganic arsenic produced lung tumors in mice (Cui, et al., 2006), while other studies found that oral exposure to DMA^V resulted in lung tumors in mice (Hayashi, et al, 1998; Kinoshita, et al., 2007) and urinary bladder tumors in rats (Wei, et al., 2002; Arnold, et al., 2006). A highly relevant study to the current project exposed CD1 mice to a "whole-life" inorganic arsenic exposure up to 24 ppm from 2 weeks prior to breeding,

during pregnancy, lactation, after weaning, and through adulthood up to 2 years. This study reported dose-dependent increases in lung adenocarcinoma, hepatocellular carcinoma, gallbladder tumors, uterine carcinomas, ovarian tumors, and adrenal tumors (Tokar, et al., 2011).

In addition to cancer, non-carcinogenic effects such as hypertension, cardiovascular disease, diabetes mellitus, and renal disease incidence rates are all increased in arsenic-exposed populations (Patel 2010, Tchounwou 2003, Guha 2008). A 2007 study of Mexican subjects concluded a dose-dependent increase in diabetes mellitus risk as arsenic exposure increases (Coronado-Gonzalez 2007). A meta-analysis from 2012 concluded that there is also a dose-dependent increase in hypertension of populations exposed to increasing exposures of arsenic (Abhyankar 2012). Due to the detrimental effects on human health from arsenic, in 2001 the U.S. Environmental Protection Agency lowered the drinking water standard for arsenic in public water systems (PWS) from 50 ppb to 10 ppb, and all PWS's were required to be in compliance with by January 23, 2006 (EPA 2001).

Arsenic and Cellular Differentiation

While the mechanisms behind arsenic-induced reductions in growth and neurological impairment are not fully understood, one possibility is that arsenic impairs the ability of stem cells to differentiate. For example, one study showed that a 14-day exposure of 10 μM sodium arsenate to human keratinocytes (SCC-9 cells) reduced expression of involucrin, a protein crucial to keratinocyte differentiation (Kachinskas 1994, Kachinskas 1997). Another study demonstrated that pluripotent C3H 10T1/2 cells exposed to 6 μM

sodium arsenite for two months reduced their ability to differentiate into adipocytes, as measured by both morphology and reduced expression of the adipocyte markers aP2, PPARγ and C/EBPα (Wauson 2002). A similar study of C3H 10T1/2 cells concluded that short-term arsenic exposures of 3, 6, and 10 μM sodium arsenite exposures showed a dose-dependent reduction in adipocyte differentiation (Trouba 2000).

In addition to reduced differentiation in keratinocytes and adipocytes, muscle and neuronal differentiation appear to be affected by arsenic exposure. A study exposing C2C12 mouse myoblasts to 20 nM arsenic found that the arsenic impaired differentiation, as measured by lower levels of myogenin transcripts and reduced multi-nucleation (Steffens 2011). In a study of muscle repair, male mice exposed to 0.5 or 5 ppm arsenic trioxide for 8 weeks had reduced muscle regeneration following a soleus muscle injury (Yen 2010). Arsenic exposure has also been shown to have detrimental effects on differentiating rat PC12 neuroblasts. Frankel and colleagues showed that a 120 hour exposure of PC12 cells to 2.5 and 5 μM sodium arsenite, decreased neurite outgrowth and complexity in a dose-dependent manner (Frankel 2009). A study exposing Neuro-2A neuroblastoma cells to 5 μM arsenic trioxide for 48 hours during differentiation found that is reduced neurite outgrowth in a dose-dependent and time-dependent manner (Wang 2010). Based on these previous studies on reduced differentiation, it is postulated that neurological and motor deficits resulting from arsenic exposure during embryogenesis can be attributed to decreased stem cell differentiation into myotubes and neurons.

Studies from our lab are novel from others in that we use pluripotent P19 embryonic stem (ES) cells to determine the mechanisms through which arsenic may alter

development. One such study from 2015 showed that arsenic exposure significantly reduced transcript and protein expression of Msx1 and Pax3, both of which are required for neural plate border specifier cell formation (McCoy 2015). The expression of NeuroD1 transcript and protein levels required for neural crest cell differentiation, were decreased, while Sox10 protein expression required for neural crest progenitor cell production was also decreased. Furthermore, nuclear translocation of proteins required for myotube formation were significantly reduced when compared to unexposed cells (McCoy 2015).

Chronic Arsenic Exposure and Resulting Formation of Cancer Stem Cells

Building on the conclusion that arsenic exposure can reduce cellular differentiation, several studies have shown that arsenic exposed fate-determined cells can be transformed to a cancer stem cell-like phenotype. A study of human keratinocytes (HaCaT cells) exposed to 50 ppb arsenic for 18 weeks found that the arsenic-exposed cells formed a multinucleated, cancer stem-like phenotype with higher rates of proliferation than passage matched controls (Huang 2013). Similarly, exposure of human urothelial (UROtsa) cells to a 0.05 μM concentration of the inorganic arsenic metabolite, monomethylarsenous acid (MMA^{III}) for 52 weeks transformed them into malignant cells, showing anchorage-independence after 24 weeks of exposure and formation of tumors in SCID mice after 52 weeks of exposure (Bredfeldt 2006). Complementing these findings, Wnek and colleagues determined UROtsa cells exposed to 50 nM (6.2 ppb) MMA^{III} experience a critical period at 12 weeks of exposure after which UROtsa cells are irreversibly transformed into malignant cells that formed tumors in immunocompromised mice (Wnek 2010). Further,

at 12 weeks of arsenic exposure, DNA methylation was unchanged when compared to control UROtsa samples; however aberrant DNA methylation was observed when these exposed cells were cultured for 12 more weeks in untreated media (Wnek 2010). A recent epigenetic study from 2018 exposing UROtsa cells to 50 nM MMA^{III} for up to 14 weeks and found that arsenic exposure leads to increased acetylation of the H3 histone and decreased acetylation of the H4 histone, both of which play crucial roles in chromatin condensation and transcriptional regulation. The authors hypothesized that altered histone acetylation was a factor in the malignant transformation of UROtsa cells following exposure to MMA^{III} (Ge 2018).

While arsenic appears to phenotypically and epigenetically transform differentiated cells to malignant cells, this alteration may be further mediated through arsenic induced transcriptional regulation of pluripotency markers. The transcription factors Sox2 and Oct4 have been shown to increase cell "stemness" in several different cancer stem cell lineages (CSC) by acting synergistically to maintain pluripotency and drive the transcription of various target genes (Boumahdi 2014, Leis 2012, Hochedlinger 2005, Rodda 2005). Sox2 has been shown to work with Oct3/4 to activate Oct-Sox enhancers, which in turn regulate the expression of Nanog, Oct3/4, and Sox2 itself (Masui 2007). Not only will Sox2 act synergistically with Oct3/4, but Sox2 can also regulate several transcription factors that control the expression of Oct3/4. Meanwhile, the forced expression of Oct3/4 will rescue the pluripotency of Sox2-null ES cells, lending credence to the idea that Sox2 helps maintain ES cells in a pluripotent state through ensuring the proper expression of Oct3/4 (Masui 2007). A chronic exposure of immortalized human urothelial cells (HUC1) to 1.0

μM arsenic trioxide for 12 months led to increased expression of Sox2, Oct4, and Nanog transcript and protein levels. Of particular interest, Sox2 was shown to be overexpressed gradually and irreversibly. Researchers then knocked-down Sox2 expression in these arsenic treated HUC1 cells leading to decreases in expression of Oct4, Nanog, CD133, and CD24, as well as a reduction in stem-like properties including invasion and chemoresistance (Ooki 2018).

Further, human bronchial epithelial (HBE) cells exposed to 1.0 μM arsenite for 15 weeks began to show significant increases in Oct4 transcript expression by week 5 of the exposure, as well as increases in Bmi1 and ALDH1 transcripts. This same study observed no significant increases in Sox2 or Notch1 expression (Xu 2012). A study of a different human bronchial epithelial cell line (BEAS-2B) exposed to 0.25 μM arsenite for 6 months reported increases in the expression of Klf4, Myc, Oct4, and Sox2 transcript and protein expression in the arsenic transformed cells (Chang 2014). Another chronic *in vitro* study exposed human epithelial stem/progenitor cells (WPE-stem) to 5 μM arsenite for 18 weeks. Interestingly, it was observed that several markers of self-renewal, including Bmi1, Notch1, and Oct4, were decreased for the first several weeks of the arsenic exposure, but all markers began to increase later. This pattern of expression was inversely related to the expression of PTEN, a tumor suppressor gene, leading the authors to conclude that arsenic exposure inhibits PTEN expression, resulting in the eventual increase of self-renewal marker expression and the formation of CSC-like cells (Tokar 2010). Collectively, these studies show that chronic arsenic exposure may induce cancer stem cell transformation by

increasing levels of pluripotency and self-renewal markers, while simultaneously reducing the expression of tumor suppressor genes.

Arsenic and Epithelial-Mesenchymal Transitions

Epithelial-mesenchymal transition (EMT) is a process in which immotile, polarized epithelial cells transform into motile, mesenchymal cells (Yang 2008). Specifically, mesoderm and neural crest formation result from EMT during embryogenesis, with the resulting cells maintaining their oliogopotentiality and ability to further differentiate (Yang 2008). In addition, the transformation into a mesenchymal phenotype facilitates the metastasis of cells from primary carcinomas (Thiery 2002). Immortalized human mammary epithelial cells (HMLEs) induced to go through EMT will acquire mesenchymal traits, express stem-cell markers more efficiently formed mammospheres, colonies in soft agar, and tumors (Mani 2008). A family of transmembrane glycoproteins known as cadherins play a key role in the maintenance of the organized structures of solid tissue. Of these cell-to-cell adhesion molecules, E-cadherin, N-cadherin, and P-cadherin are the most commonly studied (Gumbiner 1996). It is widely known that E-cadherin expression is inversely related to N-cadherin expression in aggressive tumors undergoing EMT, leading to increased tumor invasiveness and metastasis (Tran 1999, Christiansen 2006, Kang 2004). A study of human prostate tumors revealed that "cadherin switching," resulting in EMT through decreased E-cadherin expression and increased N-cadherin expression, was associated with cancer progression as well as cancer-specific death (Gravdal 2007).

Following these trends, a previously mentioned study of human bronchial epithelial (HBE) cells exposed to 1.0 μM arsenite for 15 weeks resulted in increased N-cadherin transcript and protein expression beginning at 5 weeks of exposure while E-cadherin expression was inversely decreased. Vimentin expression, another marker of EMT progression, was also shown to increase beginning at week 5 of arsenic exposure (Xu 2012). Immortalized human urothelial cells (HUC1) exposed to 1.0 μM arsenic trioxide for 12 months showed similar results, with N-cadherin and vimentin expression increasing in arsenic-transformed cells while E-cadherin decreased (Ooki 2018).

While EMT is driven by cadherins, many studies have implicated the Wnt/ β-catenin signaling pathway as the mechanism by which cadherins are regulated (Xu 2015). Cho and colleagues studied the role of CD44 in SW480 colon cancer cells and reported that EMT increased in cells overexpressing CD44 and decreased in CD44 knockdown cells. This may be attributed to the fact that CD44 is the transmembrane adhesion receptor for hyaluronic acid (HA). As the receptor for HA, CD44 is responsible for the degradation of HA, which can result in cell migration, cancer invasion, and metastasis (Ponta 2003, Nagano 2004, Rodgers 2006). It was shown that increased CD44 expression led to upregulation of N-cadherin expression, and the downregulation of E-cadherin expression. Additionally, β-catenin was shown to translocate to the nucleus of cells, resulting in transcription of EMT-inducing genes. This inhibited formation of the E-cadherin-β-catenin complex, and resulted in cell migration and invasion (Cho 2012). Further, the Wnt/ β-catenin target gene, L1CAM (L1), is known to induce stemness in cancer cells (Beck 2015). The L1 gene is capable of inducing nuclear translocation of β-catenin and decreasing E-

cadherin expression in both mammary epithelial cells as well as colorectal cancer cells (Shtutman 2006, Basu 2018). Previous studies from our lab show that P19 embryonic stem cells exposed to arsenic have repressed levels of β-catenin transcripts, while inversely showing increases of the Nanog gene, a marker of pluripotency, suggesting that arsenic exposure delays differentiation through increased pluripotency (Hong 2012).

Hypothesis and Aims

Thus, our hypothesis of the current study is that chronic, low-dose arsenic exposure delays differentiation and increases pluripotency of embryonic stem cells through the activation of an EMT-like mechanism.

Aim 1: Determine if a 32-week chronic arsenic exposure alters the differentiation of P19 stem cells

P19 mouse embryonic stem cells (ESC's) were exposed to 0 or 0.1 μM (7.5 ppb) sodium arsenite, a concentration lower than the current drinking water standard, for 32 weeks. Cells were sampled every 4 weeks for a total of 8 sampling periods. At each of these sampling points, ESC's were differentiated for a total of 9 days, first into embryoid bodies and then adult cells, through the hanging drop method. The goal of this aim was to develop a time course and determine whether exposure of stem cells to arsenic at levels below the current drinking water standard would impair their ability to differentiate.

To assess changes in differentiation over the 32-week period, embryoid bodies were imaged at day 9 of each sampling period to provide a subjective comparison of morphological changes. A profiler array was utilized to assess differential expression of a

suite of genes involved in cell-fate determination. Next, using the profiler array data, expression of select genes involved in cellular differentiation including Dcx, MyoD, and NeuroD, and the pluripotency markers Nanog, Sox2, Oct4, were quantified by qPCR. To further validate differential expression of pluripotency marker mRNA, immunohistochemistry (IHC) and immunoblotting were used to quantify protein expression of the pluripotency-associated transcription factor, Sox2.

Based on previous studies, it was expected that genes and proteins involved in differentiation would be down-regulated, and genes involved with pluripotency would be up-regulated in the arsenic-exposed samples by around week 24 of the exposure.

Aim 2: Determine mechanism through which arsenic impairs differentiation and maintains pluripotency of P19 stem cells

The expression of oxidative stress markers, Hmox1 and Nrf2, were analyzed by qPCR as it could be expected arsenic may impair differentiation through an oxidative stress mechanism. Furthermore, a process known as an epithelial-mesenchymal transition (EMT) is known to play a role in pluripotency induction. Therefore, IHC was used to quantify the expression of E- and N-cadherin, cell-to-cell adhesions molecules involved in EMT. It was predicted that expression of the mesenchymal marker, N-cadherin, would increase with the induction of pluripotency, while expression of the epithelial marker, E-cadherin, would be subsequently decreased.

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Chapter Two

A Chronic, 32-Week 0.1 μM (7.5 ppb) Arsenic Exposure Maintains Pluripotency in P19 Mouse Embryonic Stem Cells

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Abstract

Arsenic is a contaminant found in many foods and drinking water. Exposure to arsenic during the embryonic and fetal stages of development is known to decrease birth weight, stunt weight gain, and result in improper neuronal development and function. Previous *in vitro* studies that exposed progenitor cells, such as C2C12 myoblasts and Neuro-2A neuroblastoma cells, have reported that arsenic reduced their differentiation into myotubes and neuroblasts, respectively. This study exposed P19 mouse embryonic stem cells continuously to 0.1 μM (7.5 ppb) arsenic for 32 weeks, with the aim of delineating how chronic arsenic exposure impacts cellular differentiation during development. A cell lineage profiler array examining mRNA changes after 8 and 32 weeks of exposure showed that genes involved in pluripotency were increased, while those involved in differentiation were reduced. The temporal impairment of cellular differentiation was investigated by mRNA analysis of select pluripotency and neuronal differentiation markers throughout the 32 week exposure. The results show that Sox2 and Oct4 mRNA expression was increased by 1.4- to 1.8-fold in the arsenic-exposed cells, beginning at week 8. Compounding this, Sox2 protein expression was increased by 1.4-fold, starting at week 16. A prevailing hallmark of epithelial-mesenchymal transitions (EMT) is increased expression of the cell-to-cell adhesion molecule N-cadherin, which is known to be induced by Sox2. Exposure to arsenic resulted in significantly increased N-cadherin protein levels of 1.3-fold beginning at week 20 and continuing through week 32 of exposure, concurrent with increased grouping of N-cadherin positive cells at the perimeter of the embryoid body. Taken together, these results support that a chronic, low-level arsenic exposure during

development may delay neuronal differentiation and maintain pluripotency through an EMT-like mechanism.

1. Introduction

Arsenic (As) is a ubiquitous groundwater toxicant found throughout the world, including countries such as China, Mexico, and the United States, with the highest incidences of exposure seen in Bangladesh and India (Dwivedi 2015). Many people in these countries are exposed to arsenic concentrations higher than the World Health Organization (WHO) recommendation and U.S. Environmental Protection Agency (U.S. EPA) standard of 10 ppb in drinking water (EPA 2001, WHO 2015). In addition to the risk from drinking water, various vegetables, grains, and fruits are known to readily uptake arsenic. Specifically, rice and apples uptake arsenic from irrigation water and soil (Zavala 2008, Creger 1992, Yokel 2003, Morgan 2009). In adults, chronic arsenic exposure has been correlated with cancers of the skin, bladder, liver, lungs, and kidneys, as well as non-carcinogenic conditions including hypertension, diabetes mellitus, and cardiovascular disease (Tchounwou 2003, Guha 2008). Although exposure to arsenic at any age could cause negative health outcomes, embryonic arsenic exposure can cause deficits that may last a lifetime. Arsenic is able to cross the blood placental barrier of pregnant women, acting as a developmental toxicant and resulting in similar arsenic concentrations in both cord and maternal blood (Concha 1998). Consequently, epidemiological studies report increased incidences of stillbirths, reduced birth weight and weight gain (Concha 1998, Raqib 2009, von Ehrenstein 2006), altered locomotor activity, and learning deficits (Dakeishi 2006, Tsai 2003) in locations with high concentrations of arsenic in drinking water. Further, children subjected to arsenic exposures in excess of the 10 ppb standard,

show deficits in full-scale IQ, verbal IQ, and memory compared to their unexposed counterparts (Tolins 2014).

In order to delineate the mechanisms through which arsenic induces these developmental deficiencies, studies of rats have shown that arsenic is able to cross the blood brain barrier (Xi 2010). Investigators have shown that PC12 rat neuroblast cells acutely exposed to 5 μM and 10 μM arsenic had reduced neurite length and less branching compared to control samples (Frankel 2009). Another study exposed Neuro-2A mouse neuroblastoma cells to 5 μM arsenic trioxide for 48 hours during differentiation, resulting in reduced neurite outgrowth in a dose- and time-dependent manner (Wang 2010). In addition to neurodevelopmental deficits, arsenic also acts to hinder muscle development and repair. A study of mice exposed to 100 μg/l sodium arsenite in drinking water for 5 weeks concluded that arsenic exposure impairs muscle regeneration and the recovery of muscle function following cardiotoxin injury to the tibialis anterior (Zhang 2016). Similarly, a study of mice exposed to 0.5 and 5 ppm arsenic trioxide for 8 weeks in drinking water resulted in decreased myogenin expression, a reduction in myofibers, and an increase in collagen levels of arsenic exposed mice after induced necrotic injury the soleus muscle. An in-vitro component of this same study reported decreased differentiation of C2C12 mouse myoblasts after 4 days of 0.1-0.5 μM arsenic trioxide exposure (Yen 2010). Another study of C2C12 mouse myoblasts also described reduced differentiation, validated by decreased myogenin transcript level and reduced multi-nucleation following an exposure of 20 nM arsenic during 4 days of differentiation (Steffens 2011). Previous studies of P19 mouse embryonic stem (ES) cells from our lab have shown a reduction in both skeletal

myotube and neuron formation, but not proliferation, following a 0.5 μM sodium arsenite exposure (Hong 2012).

While these previous studies have shown that acute, *in vitro* arsenic exposure can reduce cellular differentiation, several studies have also shown that fate-determined cells chronically exposed to arsenic can be transformed to a cancer stem-cell phenotype. A study of human keratinocytes (HaCaT cells) exposed to 50 ppb arsenic for 18 weeks found that the exposed cells developed a cancer stem-like phenotype that showed higher rates of proliferation when compared to passage matched controls (Huang 2013). Similarly, exposure of human urothelial (UROtsa) cells to a 0.05 μM (6.2 ppb) concentration of the inorganic arsenic metabolite, monomethylarsenous acid (MMA^{III}) for 52 weeks transformed them into malignant cells, showing anchorage-independence after 24 weeks of exposure and the formation of tumors in SCID mice after 52 weeks of exposure (Bredfeldt 2006). Compounding these findings, Wnek and colleagues determined that UROtsa cells exposed to 50 nM (6.2 ppb) MMA^{III} experience a critical period at 12 weeks of exposure after which UROtsa cells are irreversibly transformed into malignant cells (Wnek 2010).

While arsenic appears able to transform differentiated cells to malignant cells, this alteration may be mediated through arsenic induced transcriptional regulation of pluripotency markers. The transcription factors Sox2 and Oct4 have been implicated in several different cancer stem cell lineages (CSC), increasing cell "stemness" by acting synergistically to maintain pluripotency and drive the transcription of various target genes (Boumahdi 2014, Leis 2012, Hochedlinger 2005, Rodda 2005). Sox2 has been shown to

work with Oct3/4 to activate Oct-Sox enhancers, which in turn regulate the expression of Nanog, Oct3/4, and Sox2 itself (Masui 2007). Not only will Sox2 act synergistically with Oct3/4, but Sox2 can also regulate several transcription factors which control the expression of Oct3/4. Meanwhile, the forced expression of Oct3/4 will rescue the pluripotency of Sox2-null ES cells, lending credence to the idea that Sox2 helps maintain ES cells in a pluripotent state through ensuring the proper expression of Oct3/4 (Masui 2007). A study of human bronchial epithelial (HBE) cells exposed to 1.0 μM arsenite for 15 weeks began to show significant increases in Oct4 transcript expression by week 5 of the exposure, while no significant increases in Sox2 or Notch1 expression were observed (Xu 2012). Another chronic *in vitro* study exposed human epithelial stem/progenitor cells (WPE-stem) to 5 μM arsenite for 18 weeks. Interestingly, it was shown that several markers of self-renewal, including Bmi1, Notch1, and Oct4, were decreased for the first several weeks of the arsenic exposure, before all markers began to increase later. This pattern of expression was inversely related to the expression of PTEN, a tumor suppressor gene, leading the authors to conclude that arsenic exposure inhibits PTEN expression, resulting in the eventual increase of self-renewal marker expression and the formation of CSC-like cells (Tokar 2010).

The purpose of the current study was to examine whether long-term arsenic exposure, below the current drinking standard of 10 ppb, altered the differentiation of P19 stem cells into neurons and myotubes, and if so, investigate the mechanisms responsible. Our results demonstrate that arsenic exposure after 8 weeks increases Sox2/Oct4 transcript expression, while decreasing doublecortin expression by 12 weeks of exposure, indicating

reduced neuronal differentiation. Further, at approximately 16 weeks of exposure, we report an overall increase in N-cadherin protein expression, as well as increased groupings of N-cadherin positive cells at the perimeter of arsenic exposed embryoid bodies. These results suggest that chronic arsenic exposure hinders the ability of neural progenitor cells to properly differentiate.

2. Methods

2.1 P19 Cell Culture

P19 mouse embryonic stem cells were obtained from Sigma-Aldrich (St. Louis, MO, USA). These are pluripotent embryonal carcinoma cells derived from a teratocarcinoma (McBurney 1993). Cells were cultured in α-MEM media containing 7.5% bovine calf serum, 2.5% fetal bovine serum, and 0.1% L-glutamine. Cells were cultured in control medium or medium containing 0.1 μM arsenic as sodium arsenite, equivalent to 7.5 ppb arsenic, for up to 32 weeks. This arsenic concentration is lower than the 10 ppb drinking water standard set by the EPA and WHO. Both the control and the arsenic exposed samples were cultured as three independent replicates (n=3 for each treatment). The cells were cultured in a humidified incubator at 37°C and 5% $C0_2$, and passaged every two to three days to maintain low confluency and avoid premature cellular differentiation.

2.2 P19 Cell Differentiation

At eight time points (once every four weeks of the 32-week exposure), the six separate cultures of cells (n=3 for each treatment) were differentiated in 1% DMSO media via the hanging drop method (500 cells/drop) (Wang 2008). After two days, individual embryoid bodies were transferred to 96-well ultralow attachment plates for another three days. Representative images of day 5 embryoid bodies were captured using a VWR VistaVision microscope camera. After imaging, embryoid bodies from one 96-well plate were pooled together, washed in PBS, and placed in 10% neutral buffered formalin overnight at 4^{o}C for immunohistochemistry analyses. A second 96-well plate was collected for RNA extraction and stored in Trizol (Thermo Fisher, Waltham, MA) at -80^{o}C, and a third 96-well plate of day 5 embryoid bodies was collected and stored in RIPA buffer (Thermo Fisher) at -80^{o}C for immunoblot analysis. An additional set of day 5 embryoid bodies were transferred to 0.1% gelatin-coated 48-well plates, and medium was refreshed every other day. On day 9, representative pictures were taken. Afterwards, half of the day 9 cells were collected and stored in Trizol at -80^{o}C for RNA extraction, while the other half were collected and stored in RIPA lysis buffer at -80^{o}C for protein extraction.

2.3 Cell Lineage Profiler Array

To initially assess changes over the continuous 32-week exposure, RNA from day 9 control and arsenic-exposed P19 cells from two selected time points were analyzed using a Qiagen RT^{2} Profiler Cell Lineage PCR Profiler Array (Germantown, Maryland). This array detects expression of a suite of 84 genes involved in cellular differentiation. Briefly,

RNA from week 8 and week 32 cells at day 9 of differentiation was extracted using Trizol, and RNA concentration and purity were determined on a Nanodrop (Thermo Scientific, Waltham, MA). A Qiagen RT^2 First Strand Kit was used to synthesize cDNA from 2 μg of pooled RNA from control and arsenic-exposed treatments, respectively (n=3-4). Quantitative PCR was then conducted with SYBR Green PCR Master Mix (Applied Biosystems). Array data was analyzed using Qiagen GeneGlobe Data Analysis Center (https://geneglobe.qiagen.com/us/analyze/).

2.4 Gene expression analysis using Quantitative PCR

RNA was extracted using Trizol, and RNA concentration and purity were determined on a Nanodrop (Thermo Scientific, Waltham, MA) and stored at -80°C. Reverse transcription was conducted at 37°C using 2 μg RNA, 10mM deoxynucleotide mix, 50 ng/μL random hexamers, and 1U murine moloney leukemia virus reverse transcriptase (MMLV-RT). Gene expression was determined by quantitative PCR (qPCR) on an Applied Biosystems StepOnePlus Real-Time PCR system (Foster City, CA, USA). qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems), 40ng cDNA, and 10 μM forward and reverse primers for genes of interest (Table 1). All samples were run in triplicate and melt curves were used to verify gene specific binding. PCR efficiency and linearity were determined using a standard curve generated by serial dilutions of five concentration points (10^{-3} to 10^{-7} ng DNA) of the gene of interest. Gapdh was used as a housekeeping gene, as we have shown previously that its expression does not vary due to arsenic exposure (Liu 2018). Relative fold expression compared to the

controls was quantified by the Delta-Delta Ct method (Schmittgen 2008). Statistical significance of differential gene expression was determined by comparing the average 2^ddCt results of the control and arsenic exposed replicates using Student's t-test ($p \leq 0.05$) (n=3-4)**.**

2.5 Determining Protein Expression by Immunohistochemistry

After an overnight fixation, day 5 embryoid bodies were dehydrated, embedded in paraffin, and sectioned at 5 μm. Slides were deparaffinized and antigen retrieval was carried out in Tris-EDTA buffer (pH 9). Primary antibodies for Sox2 (1:2000, Abcam, #ab97959), N-cadherin (1:250, Santa Cruz, #sc-59987), and E-cadherin (1:250, Novus, #NBP2-19051) were incubated overnight at 4^{o}C. Following incubation with Alexa Flour 488 secondary antibodies (1:1000, anti-rabbit, #A11034 and 1:500, anti-mouse, #A11001); Life Technologies), nuclei were counterstained with DAPI (Invitrogen). All samples were imaged on a Leica SPE Confocal microscope (Wetzlar, Germany) equipped with traditional PMT detectors and a 20x objective. Excitation wavelength for all antibodies was 488 nm with emission wavelength between 500-560 nm, while the excitation wavelength for DAPI was 405 nm with an emission wavelength of 430-480 nm. Imaging parameters (laser power, gain, and offset) for each antibody were held consistent between control and arsenic-exposed groups at each individual time point for each antibody. Thus, statistical significance was determined by comparing control and arsenic-exposed cells of the same time point. Images were analyzed in ImageJ to determine any differences in protein expression (pixel intensity) (Tenbaum 2012). Sox2 expression was determined by

counting the number of cells using the particle analyzer function, and then dividing the integrated density by cell count to get an integrated density value (IDV) per cell. This analysis was done to account for differences in embryoid body sizes. Since N-cadherin was predominantly expressed at the peripheral boundaries of embryoid bodies, protein expression in ~5 cells of the outer border only were included in the analysis. The outer edge of the embryoid body and an inner ring were traced, and fluorescence within these borders was analyzed. This reduced variability due to non-uniform embryoid body size and/or size of the N-cadherin negative cell mass within embryoid bodies. Using this measurement, an IDV value could be determined. Expression of E-cadherin was more evenly spread though embryoid bodies, therefore expression was analyzed by the IDV of each embryoid body. All data were averaged for each treatment group, and statistical differences between the control and arsenic exposed replicates of each time point were determined using Student's t-test ($p \leq 0.05$) (n=3-4).

2.6 Analyzing Sox2 Expression by Immunoblotting

Cells were exposed to 0 or 0.1 μM arsenic, collected at day 5 and day 9 of differentiation, and stored in RIPA lysis buffer (Thermo Fisher) at -80 ^{o}C. Day 5 and 9 cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Thermo Fisher), and then centrifuged at 12,000 g for 20 minutes. Protein concentration of the supernatants was determined using a BCA protein assay kit (Thermo Scientific), with BSA as a standard. Proteins (10 μg) were electrophoresed onto 5-20% TBX gels (Bio-Rad, Hercules, CA). Immunoblotting was performed according to standard methods, using

primary antibody for Sox2 (1:1000, Abcam, #ab97959). Secondary antibody and dilution was goat anti-rabbit-HRP (1:2000, Santa Cruz, #sc-2004). Protein expression was examined by chemiluminescence (Luminol, Santa Cruz) on a Bio-Rad ChemiDoc imaging system. Densitometry values were determined using ImageJ software and normalized to Gapdh (1:1000, GeneTex, #GTX627408) as a loading control. Densitometry values for each treatment were averaged and statistical differences between the control and arsenic exposed replicates were determined using Student's t-test ($p \leq 0.05$) (n=3-4).

3. Results

3.1 Chronic, low-level arsenic exposure impairs cellular differentiation

The overarching goal of this study was to assess the impacts of long-term, low-level arsenic exposure on cellular differentiation. Thus, P19 mouse embryonic stem cells were chronically exposed to 0.1 μM (7.5 ppb) arsenic for up to 32 weeks. Every four weeks, a portion of the cells were differentiated via hanging drops and DMSO treatment (Wang 2008), and their development into embryoid bodies (EBs) after 5 days (D5) and then terminally differentiated cells after 9 days (D9) was examined. Images taken of day 9 embryoid bodies (Figure 1) indicate that there does not appear to be any morphological differences between the control and arsenic-exposed cells at any of the time points examined, as both are differentiating into elongated myoblasts and neuroblasts.

To better understand if a low-level, chronic arsenic exposure resulted in impairment of cellular differentiation, mRNA levels of a suite of 84 genes were examined using a mouse cell lineage PCR array. Analysis of differential gene expression in the week 8 and

week 32 cells indicates that transcript levels of genes involved in maintaining pluripotency, including Sox2 and Oct4 were upregulated in the arsenic-exposed cells (Table 2). Similarly, transcripts of ectodermal and neuroectodermal germ cells were increased in the arsenic-exposed cells. These genes included Fgf5, Forkhead box D3 (FoxD3), zinc finger protein of the cerebellum 1 (Zic1), and gastrulation brain homeobox 2 (Gbx2). However, genes that were indicative of more differentiated neurons, including doublecortin (Dcx) and prominin 1 (Prom1), were both decreased in the arsenic exposed cells (Table 2). Genes involved in mesoderm formation, such as brachyury (Tbxt) and Gata2 were either downregulated or not changed, while those involved in terminal skeletal muscle differentiation were reduced (Table 2). Genes involved in endoderm formation were either not expressed or were expressed at low levels, but not different between the groups (data not shown).

Using this data, expression of select differentially-expressed genes were examined in P19 cells exposed acutely (9 days) to 0.5 μM arsenic, to ensure the responses were due to arsenic exposure, rather than long-term culture. Transcripts required for the maintenance of pluripotency, such as Sox2 and Oct4 (Takahashi 2006) were examined, along with markers for neuronal progenitors and immature neurons, including Gad2 and doublecortin (Patel 2006, Francis 1999). In the 0.5 μM arsenic-exposed cells, Sox2 and Oct4 expression remained unchanged, while Gad2 expression decreased by 2.5-fold and doublecortin expression decreased by 5-fold (Figure 2).

Next, differentiation expression changes in the chronically-exposed cells was examined. Since both Sox2 and Oct4 transcription factors are involved in pluripotency

maintenance, and each protein can bind to and feedback on the others promoter to enhance gene expression (Rodda 2005, Masui 2007), the expression of Oct4 and Sox2 were averaged. At week 0, there are no differences in Sox2/Oct4 expression. However, their expression gradually increased in the cells chronically exposed to 0.1μM arsenic such that at week 8, they were 1.7-fold higher than in the control cells (Figure 3). Similarly, in weeks 12 and 20, Sox2/Oct4 expression was still significantly increased by 1.6-fold and 1.4-fold, respectively (Figure 3). While no statistical differences were seen between the week 24 samples, relative levels in the exposed cells were increased by ~1.5 fold (Figure 3).

In contrast, transcript levels of doublecortin (Dcx) were reduced between weeks 8 and 24 (Figure 4). The doublecortin gene is highly expressed in the developing brain, specifically in migrating neurons (Francis 1999). While Dcx expression was consistently reduced during the chronic exposure, this reduction was only statistically significant at week 12 and week 20 with fold changes of 1.3- and 1.4 -fold, respectively (Figure 4). Taken together, these results suggest that chronic, low-level arsenic exposure represses neural differentiation and allow cells to remain in a more pluripotent state

3.2 Arsenic exposure increases the expression of Sox2 and N-cadherin over time

To confirm changes in pluripotency, Sox2 protein expression was examined by immunohistochemistry at weeks 0, 8, 16, 20, and 28. While some Sox2 was present in the cytoplasm, the majority was observed to be in the nucleus of individual cells (Figure 5A). No differences in Sox2 expression were seen between control and arsenic-exposed cells at week 0 or week 8 (Figure 5A). However, overall Sox2 expression was increased by week

16 and remained elevated in cells chronically exposed to arsenic. Image quantification showed that arsenic-exposed cells had increased expression of Sox2 by 1.4-fold at week 16 and by week 28, the expression level had increased even further to 2.1-fold higher than controls (Figure 5B).

Previous studies have described a feedback loop between Sox2 overexpression and the induction of epithelial-mesenchymal transitions (EMT), possibly leading to cellular dedifferentiation (Yang 2014, Herreros-Vilanueva 2013). To examine a possible EMT-like mechanism of maintained pluripotency, the EMT marker N-cadherin, was investigated by immunohistochemistry. Subsequent image analysis indicated that chronic arsenic exposure results in a temporal increase in the expression of N-cadherin (Figure 6A). In addition, N-cadherin positive cells clustered together at the periphery of embryoid bodies, demonstrating arsenic-induced protein patterning changes. While there were no differences in N-cadherin expression early in the exposure, arsenic-exposed cells at weeks 20 and 28 showed significant increases in N-cadherin levels (Figure 6B). The expression of E-cadherin was also examined, however in the current study it was shown that E-cadherin protein expression remained unchanged through the exposure (Figure 6C-5D).

4. Discussion

The results demonstrate that a chronic, environmentally-relevant arsenic exposure hinders cellular differentiation and maintains stemness through the induction of markers commonly indicative of epithelial-mesenchymal transition (EMT). This increased pluripotency appears to result in decreased neural differentiation.

4.1 Arsenic maintains pluripotency of neural stem cells

To investigate arsenic-impaired differentiation, the pluripotency-associated transcription factors, Nanog, Sox2, and Oct4, were assessed. A known positive feedback loop exists between Sox2 and Oct4, whose expression is directly tied to increased pluripotency (Rodda 2005, Masui 2007). Though well-known to play a role in the general pluripotency maintenance of totipotent cells, Sox2 is also commonly expressed in multipotent lineages including embryonic neural stem cells (Ferri 2004). Combined Sox2 and Oct4 transcript levels were consistently elevated in embryonic stem cells chronically exposed to arsenic, with a significant increase of 1.7-fold first observed at week 8 of exposure. Protein expression changes of Sox2 observed by immunohistochemistry and immunoblotting were much more pronounced. Sox2 levels assessed by immunohistochemistry began to be up-regulated later than Sox2/Oct4 mRNA, with increases of 1.5- and 2.1-fold in arsenic treated samples at week 20 and week 28 of exposure, respectively. It was also noted that Sox2 remained localized in the nuclei of cells, with minimal translocation. Increased expression of Sox2 protein levels by immunoblot of 10-fold at week 20 and 15-fold at week 24 compound these findings. Robust and consistently increased Sox2 protein expression, paired with temporally variable expression of Sox2/Oct4 mRNA, suggest that arsenic may be acting upon a possible epigenetic mechanism to maintain pluripotency in stem cells. Meanwhile, Nanog expression was not consistently altered throughout the chronic 0.1 μM arsenic exposure (data not shown). Similarly, a previous acute study of 0.1, 0.5, and 1 μM arsenic on P19 stem cells resulted

in significantly increased mRNA expression of Nanog at the 0.5 and 1 μM arsenic concentrations, but not at 0.1 μM (Hong 2012).

Several previous studies have implicated arsenic exposure in the induction of Sox2 and Oct4. A chronic exposure of 0.25 μM arsenic for six months reprogrammed human bronchial epithelial cells (BEAS-2B) into cancer stem cells (CSC's) that exhibited elevated Sox2 and Oct4 expression levels, while a study of human urothelial cells (HUC1) chronically exposed to 1 μM arsenic trioxide for up to 12 months attained malignant properties which included increased Sox2 expression, self-renewal, invasion, and chemoresistance (Chang 2014, Ooki 2018). It is not being proposed that the current study resulted in the formation of CSC's, however these previous studies do show differential expression of Sox2 and Oct4 similar to that observed in this chronic exposure and consistent with arsenic-induced pluripotency.

In order to determine possible downstream consequences of maintained pluripotency, the expression of several early neural markers were investigated. Previous studies from our lab have shown a reduction of transcription factors involved in neural differentiation, including neurogenin, Ascl1, and NeuroD1 following a 0.5 μM arsenic exposure (Hong 2012, Liu 2014, Liu 2018). However, being that these markers were not altered at the 0.1 μM concentration, several new genes of interest were examined. These included Gad2, a gene associated with synaptic vesicles and GABA production; and doublecortin (DCX), a marker of immature neurons often expressed during developmental neuronal migration (Patel 2006, Francis 1999). Expression of Gad2 was observed to be decreased by 2-fold in an acute 0.5 μM exposure and down-regulated at both week 8 and

week 32 of the chronic, 0.1 μM exposure. Further, a significant 5-fold decrease in doublecortin mRNA was observed in the acute 0.5 μM exposure, while expression was consistently reduced throughout the chronic, 32-week, 0.1 μM exposure with statistically significant decreases of ~1.3- and ~1.4 -fold at weeks 12 and 20, respectively. These findings were congruent with a study that reported doublecortin (DCX) expression to be decreased in mice exposed to 50 ppb arsenic during perinatal development (Tyler 2013).

Overall, the current study demonstrates that Sox2 and Oct4 expression increased over time in arsenic-exposed cells, concomitant with a reduction in Gad2 and doublecortin expression. These results suggest that neural pluripotency is maintained in embryonic stem cells following chronic arsenic exposure.

4.2 Arsenic alters N-cadherin protein expression and patterning within embryoid bodies

During proper embryonic development, the expression of a family of cell-to-cell adhesion molecules known as cadherins, are highly regulated. In particular, epithelial and neural cadherins (E- and N-cadherin, respectively) have been implicated in developmental EMT and consequently regulate embryonic gastrulation and neurulation (Stemmler 2008, Brayshaw 2016). Following chronic arsenic exposure, the expression of N-cadherin and E-cadherin were assessed by immunofluorescence. It was shown that N-cadherin protein expression was first increased in the arsenic-treated samples at week 20 by 1.3-fold. Being that N-cadherin is a cell-cell adhesion molecule, the N-cadherin antibody was predominantly expressed at the periphery of embryoid bodies of both control and arsenic-

exposed samples as they expanded and grew. Further, it was observed that N-cadherin positive cells of the arsenic-treated samples clustered together, resulting in altered patterning which was less pronounced in control samples.

Interestingly, it has been reported that N-cadherin is required for the maintenance of neural precursors in the ventricular zone of the developing cerebral cortex, with decreases in expression resulting in premature neural differentiation and increased cellular migration from the ventricular zone (Zhang 2010). Increased N-cadherin expression and grouping in the arsenic-exposed cells could be indicative of maintained stemness and delayed differentiation of neural progenitor cells. A recent study of lead (Pb) toxicity in P19 cells reported that exposures of 1-3 μM increased Sox2 expression while N-cadherin levels were reduced, which corresponded with decreased neuronal and glial differentiation (Mansel 2019). While the current arsenic exposure also observed increased Sox2 expression, the increased expression of N-cadherin contradicts the study of lead exposure. However, this could be explained by the possibility of the formation of different neural stem cell populations in each study. It has been shown that N-cadherin is expressed in neuroblasts and neuroblast transit-amplifying cells, while down-regulation of N-cadherin promotes glial cell differentiation (Yagita 2009). In the current study, arsenic may be acting to maintain neuroblast and neuroblast transit-amplifying lineages, at the cost of glial differentiation.

Typically, there is an inverse relationship in the expression of N-cadherin and E-cadherin during EMT due to a process known as cadherin switching. During cadherin switching, expression of the epithelial-associated E-cadherin is reduced, while expression

of the mesenchymal-associated N-cadherin is increased, leading to the promotion of cellular motility and invasion. This process is reversible and known to play a crucial role in proper embryonic development as well as cancer formation (Stemmler 2008, Brayshaw 2016, Maeda 2005). Interestingly, throughout the chronic arsenic exposure, as N-cadherin expression was temporally increased, E-cadherin levels remained static.

Of the common EMT markers, it is known that N-cadherin in particular may be regulated via Sox2, as N-cadherin expression is thought to be governed by several Sox-dependent enhancers (Matsumata 2005). Beyond its required role in the formation of a pluripotent inner cell mass early in development, it has been widely shown that Sox2 is involved in the induction of EMT, as well as stem cell and cancer stem cell maintenance and proliferation (Avilion 2003, Herreros-Villanueva 2013, Novak 2019, Weina 2014). It has been shown that Sox2 overexpression promotes an EMT phenotype in laryngeal cancer Hep-2 cells through decreased expression of E-cadherin, and increased expression of mesenchymal markers N-cadherin, vimentin, fibronectin, and α-SMA (Yang 2014). Likewise, in a study of urothelial cells, long-term arsenic exposure led to increased Sox2 expression, concurrent with the aforementioned hallmarks of EMT; decreased E-cadherin, and increased N-cadherin and vimentin expression (Ooki 2018).

These previous studies, combined with the findings of the current study, suggest that arsenic may induce N-cadherin expression through increased Sox2 levels, maintaining neural stem cell pluripotency through an EMT-like mechanism.

5. Conclusion

A chronic arsenic exposure of 0.1 μM inorganic arsenic for 32 weeks was shown to impair cellular differentiation and induce an EMT-like phenotype in P19 mouse embryonic stem cells. Consistently elevated Sox2 protein and Sox2/Oct4 mRNA expression, in conjunction with decreased Gad2 and doublecortin levels is indicative of pluripotency maintenance and diminished differentiation. Further, arsenic exposure led to temporally increased protein expression and altered patterning of the EMT marker, N-cadherin. Taken as a whole, chronic, low-level arsenic exposure appears to utilize an EMT-like mechanism to impair neural differentiation and maintain pluripotency through delayed neuroblast migration. This notion is supported by increased expression of N-cadherin, known to be implicated in hindering neuroblast migration; as well as decreased expression of the neuronal migration marker, doublecortin (Zhang 2010, Francis 1999).

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Figure legends

Fig. 1. Effect of arsenic on stem cell morphology through 32 weeks of exposure. Shown are representative images of day 9 control and 0.1 μM (7.5ppb) arsenic-exposed differentiated cells at weeks 0, 16, and 32 of exposure. Arrows show elongating and differentiating cells.

Fig. 2. Effect of arsenic exposure on neural, pluripotency, and oxidative stress-related transcripts. (A.) Transcript expression following a nine day acute exposure to 0.5 μM (37.5ppb) arsenic exposure was quantified by qPCR. Data was normalized to Gapdh and is expressed as the average 2^ddCt for each group (n=3 flasks). Values of control and arsenic-treated samples were compared at each respective time point, and all expression fold changes used week 0 control samples as the reference. Statistical differences (*) were determined using Student's t-test ($p<0.05$). (B.) Transcript expression following a nine day acute exposure to 0.1 μM (7.5ppb) arsenic exposure was quantified by qPCR. Data was normalized to Gapdh and is expressed as the average 2^ddCt for each group (n=3 flasks). Values of control and arsenic-treated samples were compared at each respective time point, and all expression fold changes used week 0 control samples as the reference. Statistical differences (*) were determined using Student's t-test ($p<0.05$).

Fig. 3. Arsenic exposure increases Sox2/Oct4 transcript expression. Transcript levels were quantified by qPCR. Independent data sets of Sox2 and Oct4 were normalized to Gapdh, then respective fold changes were averaged together and expressed as the mean 2^ddCt for each group (n=3 flasks). Values of control and arsenic-treated samples were compared at each respective time point, and all expression fold changes used week 0 control samples as the reference. Statistical differences (*) were determined using Student's t-test ($p<0.05$).

Fig. 4: Arsenic exposure reduces the transcript expression of the neural progenitor gene Doublecortin. Transcript expression of Doublecortin was quantified by qPCR. Data was normalized to Gapdh and is expressed as the average 2^ddCt for each group (n=3 flasks). Values of control and arsenic-treated samples were compared at each respective time point, and all expression fold changes used week 0 control samples as the reference. Statistical differences (*) were determined using Student's t-test ($p<0.05$).

Fig. 5: Sox2 expression increased resulting from arsenic exposure. (A.) Representative images of Sox2 expression in day 5 control and 0.1 μM (7.5ppb) arsenic exposed embryoid bodies at weeks 0, 8, 16, 20, 28, and 32 of exposure. (B.) The integrated density function in ImageJ was used to assess relative fluorescence (n=3-4 per exposure group) at 20x magnification. Statistical differences (*) were determined using Student's t-test ($p<0.05$). (C.) The levels of Sox2 protein expression were determined by immunoblotting at week 20 and week 24 of arsenic exposure, with representative blots (n=4) shown. Sox2 specific antibodies were used to detect total protein levels. (D.) Sox2 protein levels at week 20 and

week 24 were normalized to Gapdh and relative expression (n=4 per exposure group) was assessed using the integrated density function in ImageJ. Statistical differences (*) were determined using Student's t-test ($p<0.05$).

Fig. 6: Arsenic exposure results in increased N-cadherin expression and grouping of N-cadherin positive cells. (A.) Representative images of N-cadherin expression in day 5 control and 0.1 μM (7.5ppb) arsenic exposed embryoid bodies at weeks 0, 8, 16, 20, 28, and 32 of exposure. (B.) The integrated density function in ImageJ was used to assess relative fluorescence (n=3-4 per exposure group) at 20x magnification. Statistical differences (*) were determined using Student's t-test ($p<0.05$). (C.) Representative images of E-cadherin expression in day 5 control and 0.1 μM (7.5ppb) arsenic exposed embryoid bodies at weeks 0, 8, 16, 20, 28, and 32 of exposure. (D.) The integrated density function in ImageJ was used to assess relative fluorescence (n=3-4 per exposure group) at 20x magnification. Statistical differences (*) were determined using Student's t-test ($p<0.05$).

Table 2.1: Genes of interest quantified by qPCR.

Gene	**Sequence**		**Melting Temp. (ºC)**	**Function**
	Forward	**Reverse**		
Gapdh	5'-tgcgacttcaacagcaactc-3'	5'-atgtaggccatgaggtccac-3'	56	Normalization
Sox2	5'-aggcagagaagagagtgtttg-3'	5'-cttctctcctttctttctctctcc-3'	55	Pluripotency
Oct4	5'-cctacagcagatcactcacatc-3'	5'-tgtccctgtagcctcatact-3'	58	Pluripotency
Gad2	5'- gtctgcttctggtttgtacct-3'	5'-gtggtcccatactccatcattc -3'	56	GABA-production
Doublecortin (DCX)	5'-ggtgacggatgaatggactt-3'	5'-cgtaccttcttggccttctt-3'	56	Neural Differentiation
Nrf2	5'-ctccgtggagtcttccatttac-3'	5'-gcactatctagctcctccatttc-3'	58	Oxidative Stress
Heme Oxygenase (Hmox)	5'-gtacacatccaagccgagaa-3'	5'-tggtacaaggaagccatcac-3'	58	Oxidative Stress

Table 2.2 Cell lineage profiler array differential expression of select genes

	Week 8	Week 32	
Gene symbol and function	**Expression in arsenic/control cells**	**Expression in arsenic/control cells**	**Change in arsenic-exposed cells**
Pluripotency			
Sox2	1.9	3.3	↑
Oct4	2.7	0.7	↑
Nanog	0.8	0.8	–
Ectoderm and neuroectoderm germ cells			
Fgf5	2.2	1.4	↑
FoxD3	1.5	1.2	↑
Otx2	1.5	0.5	–
Zic1	1.5	1.0	↑
Gbx2	3.7	1.3	↑
Differentiated neurons			
Dcx	0.1	0.4	↓
Prom1	0.1	0.7	↓
Gad2	0.1	0.1	↓
Mesoderm germ cells			
Gata2	0.6	1.8	–
Tbxt	0.1	0.6	↓
Differentiated muscles			
Myh1	ND	0.1	↓
Myh7	3.3	0.6	–

ND = not detectable

Fig. 1

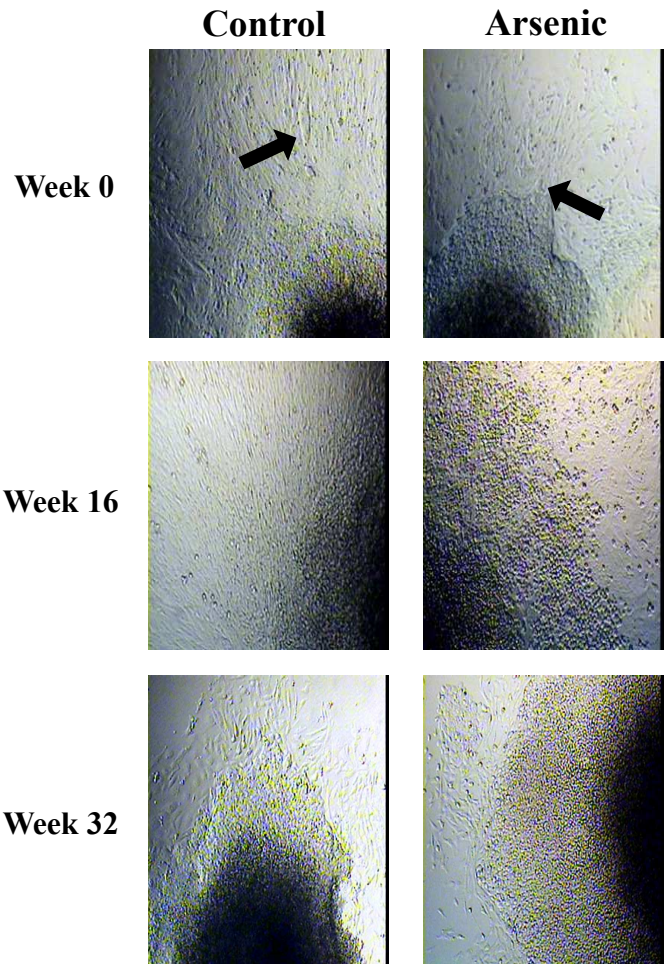
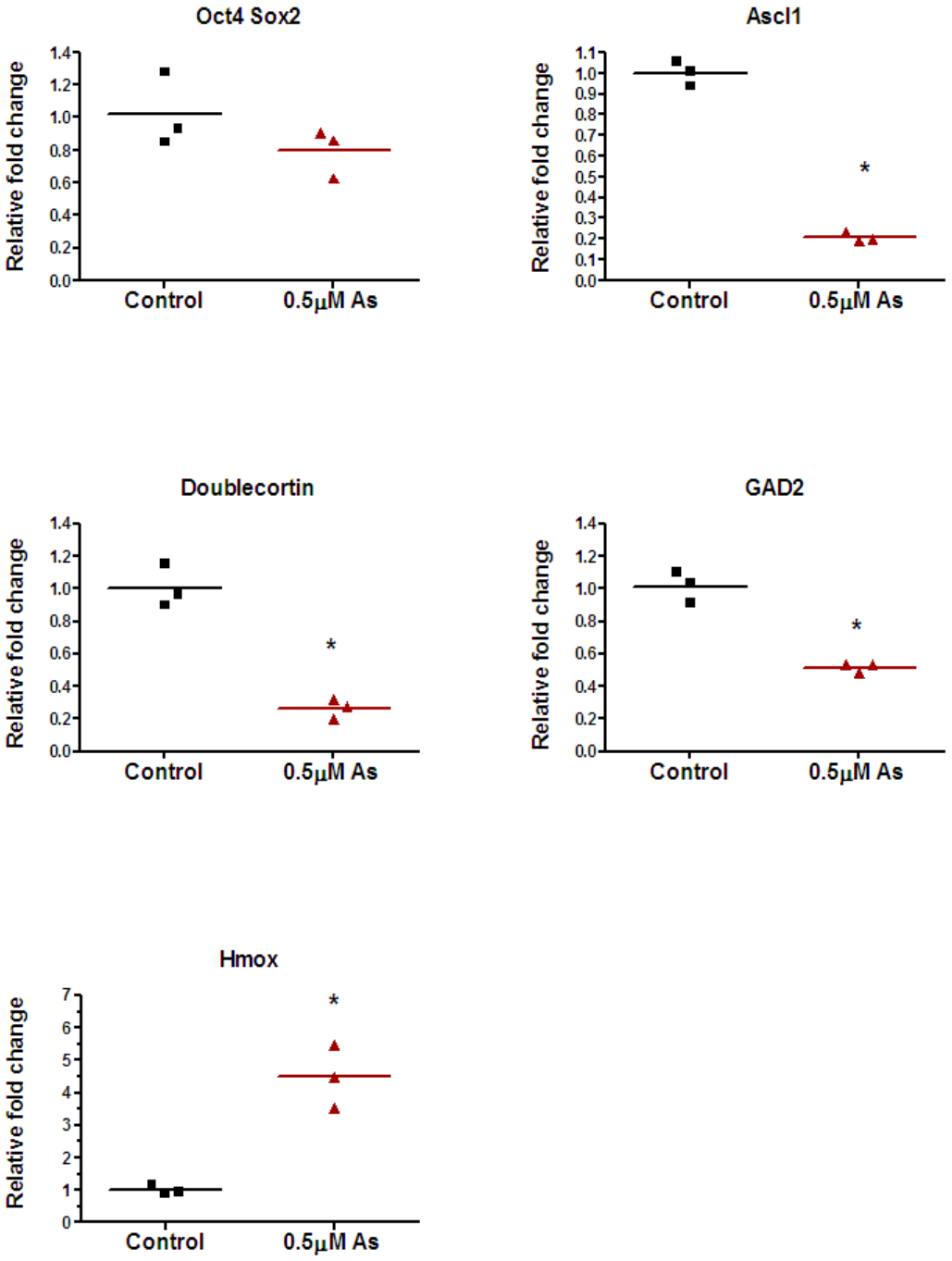
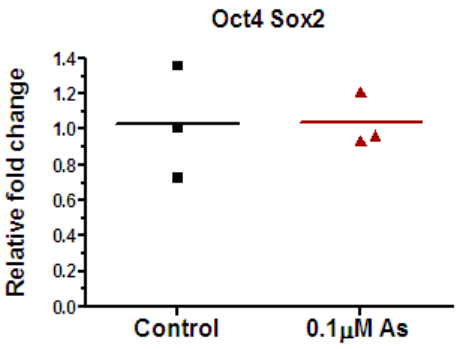


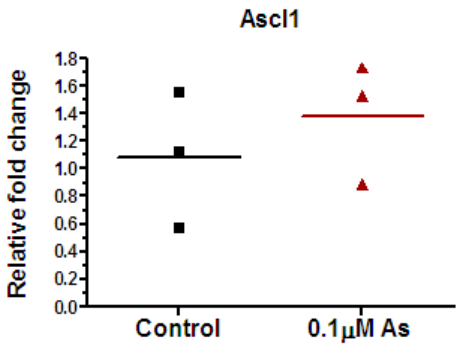
Fig. 2

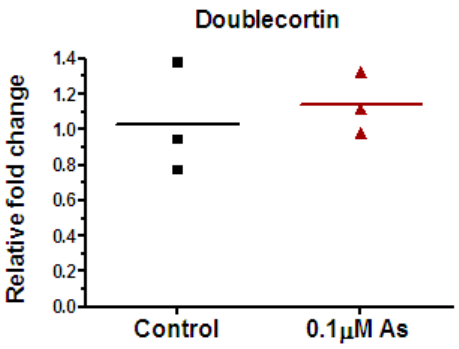
A.

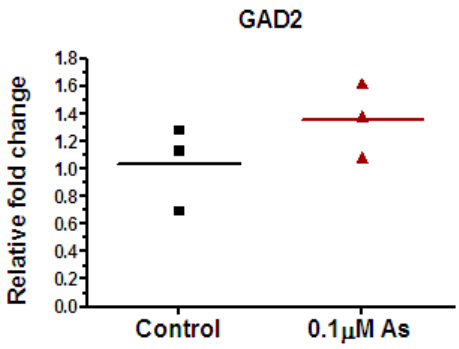

Oct4 Sox2
Relative fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.5μM As
Ascl1
Relative fold change
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
0.5μM As
Doublecortin
Relative fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
0.5μM As
GAD2
Relative fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
0.5μM As
Hmox
Relative fold change
7
6
5
4
3
2
1
0
*
Control
0.5μM As

B.


Oct4 Sox2
Relative fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.1μM As


Ascl1
Relative fold change
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.1μM As


Doublecortin
Relative fold change
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.1μM As


GAD2
Relative fold change
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.1μM As

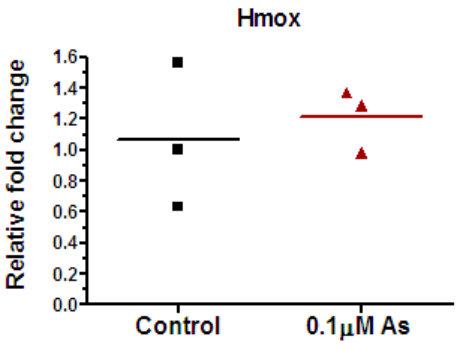

Hmox
Relative fold change
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
0.1μM As

Fig. 3

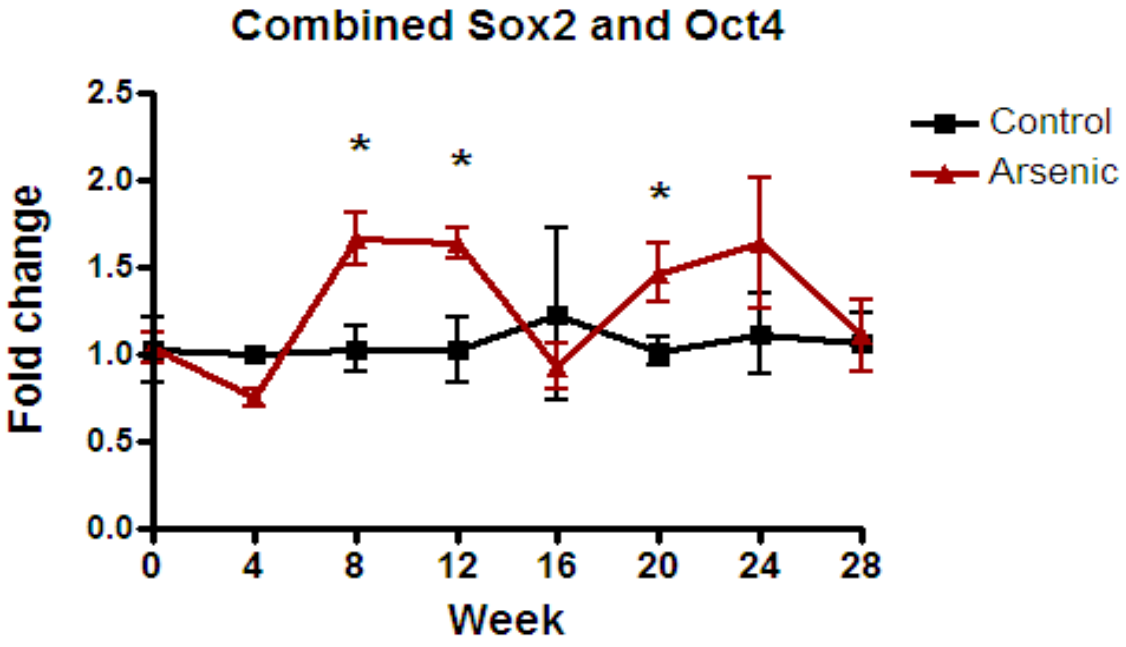

Combined Sox2 and Oct4
Fold change
2.5
2.0
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
28
Week
*
*
*
Control
Arsenic

Fig. 4

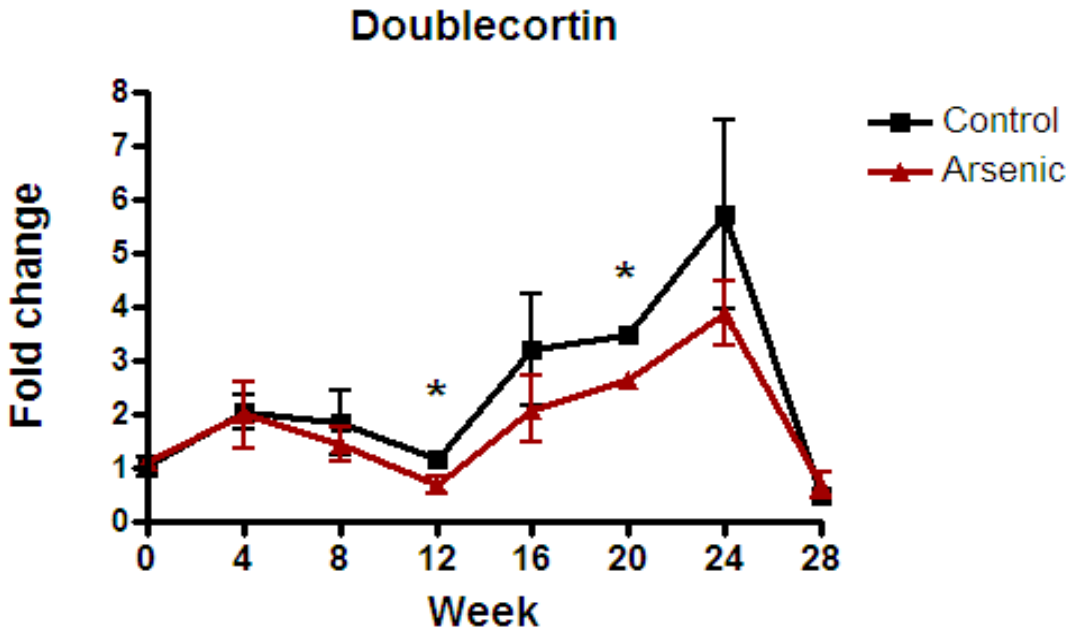
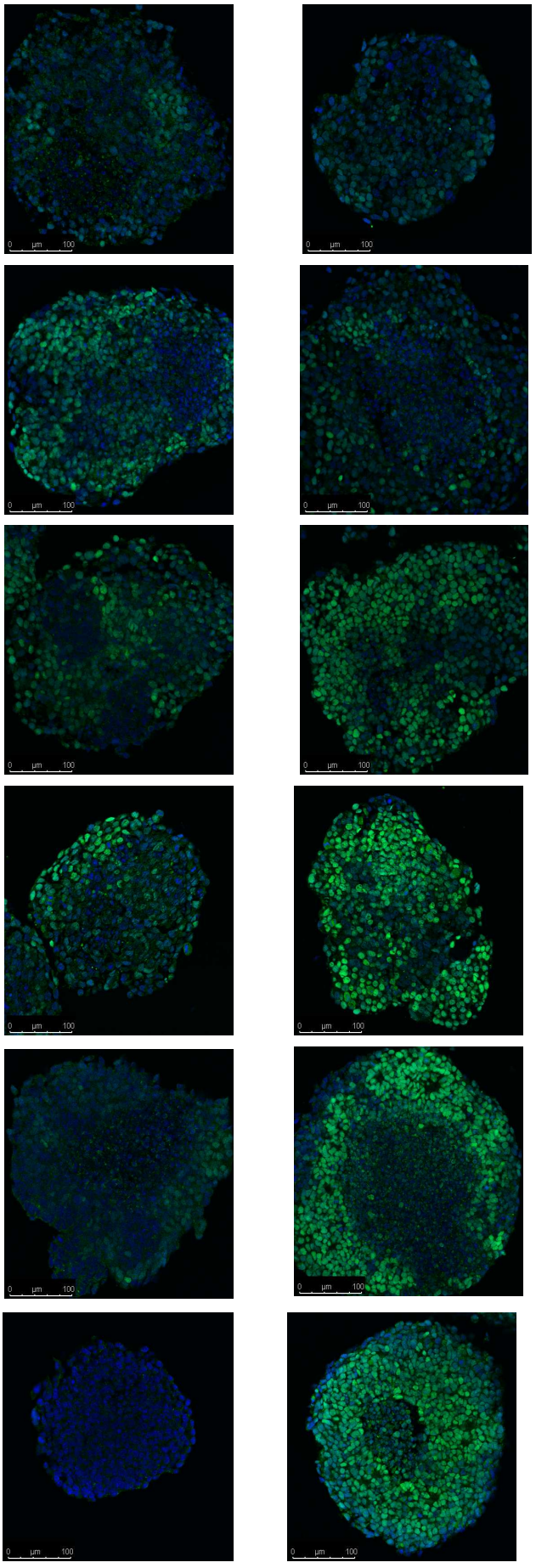
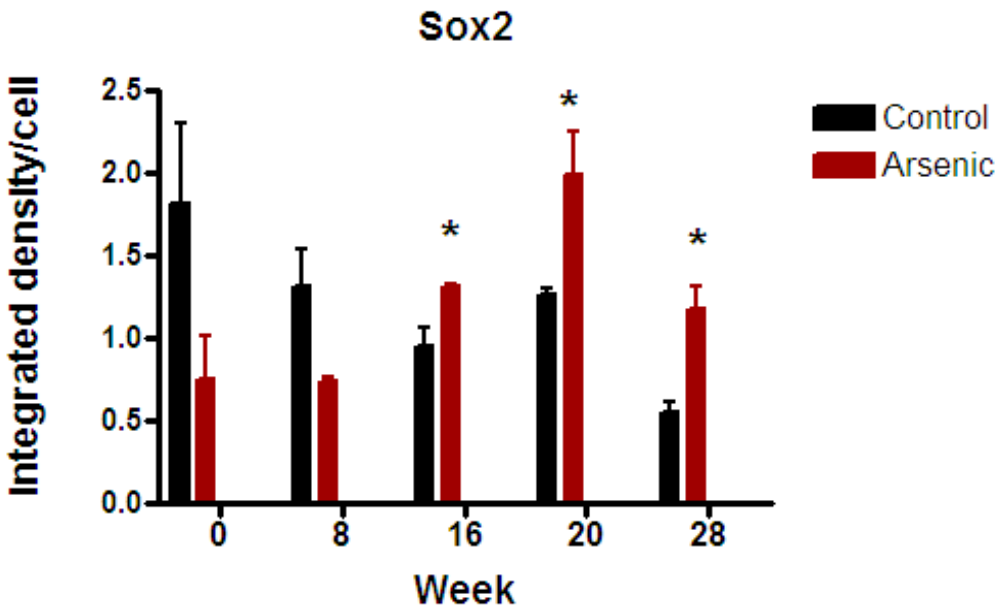

Doublecortin
Fold change
Week
Control
Arsenic
0
1
2
3
4
5
6
7
8
0
4
8
12
16
20
24
28
*
*

Fig. 5

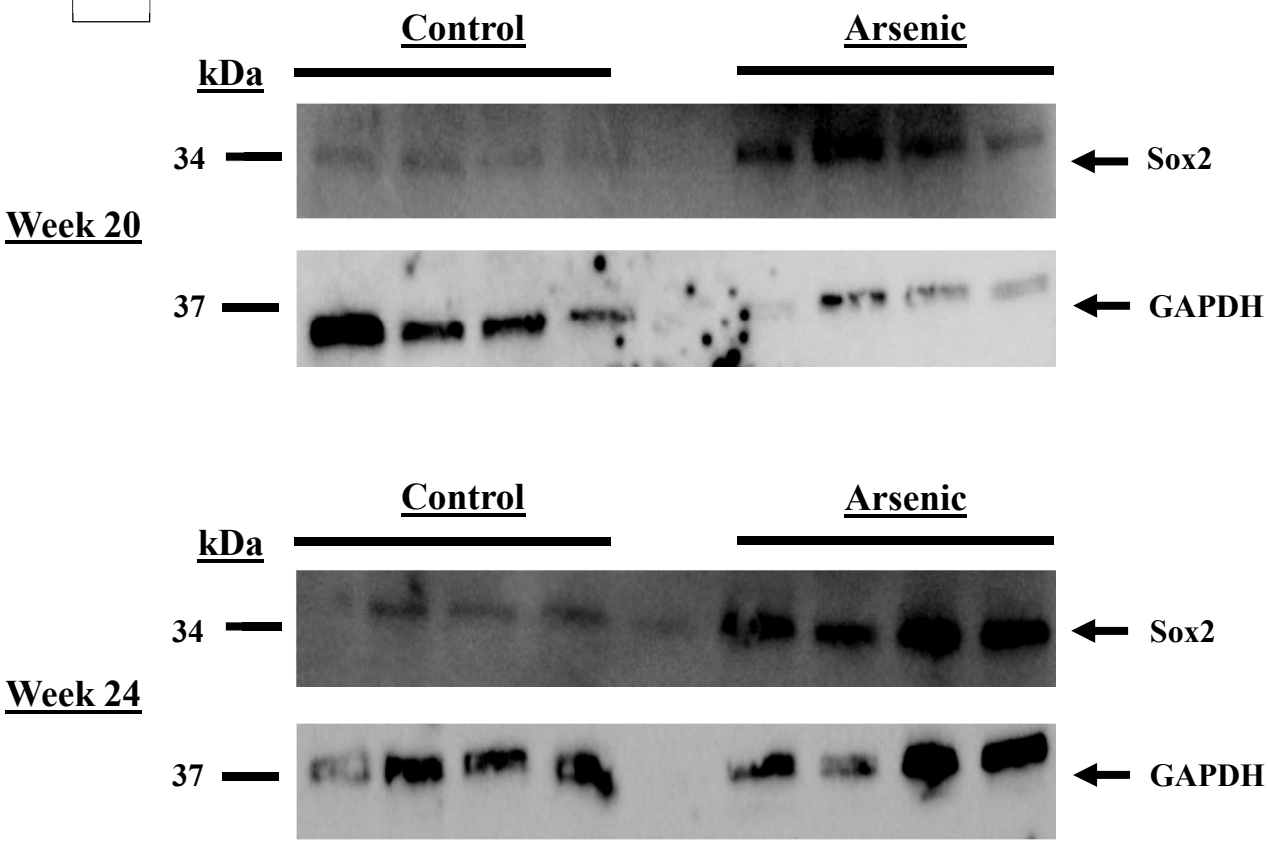
A.



B.


Sox2
Integrated density/cell
2.5
2.0
1.5
1.0
0.5
0.0
0
8
16
20
28
Week
*
*
*
Control
Arsenic

C.


Control
Arsenic
kDa
34
Sox2
Week 20
37
GAPDH
Control
Arsenic
kDa
34
Sox2
Week 24
37
GAPDH

D.

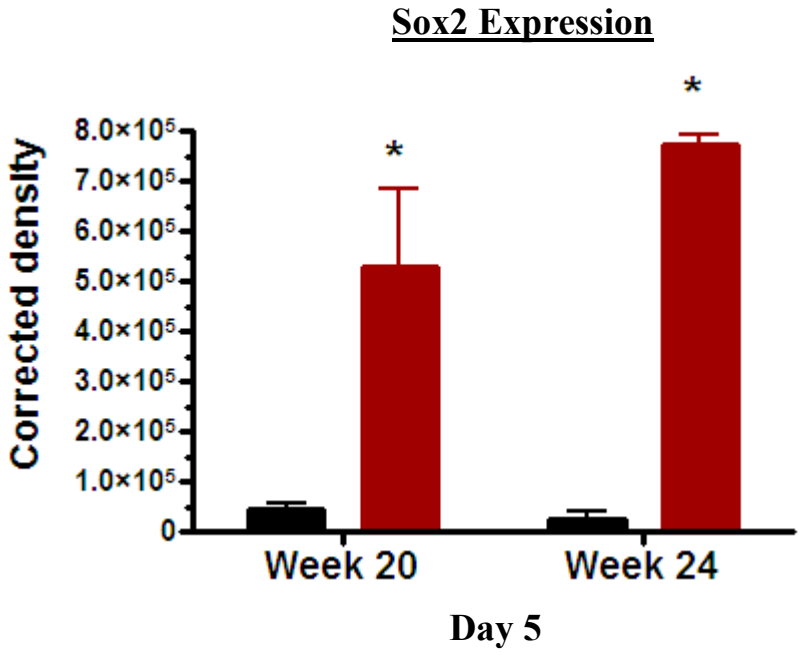
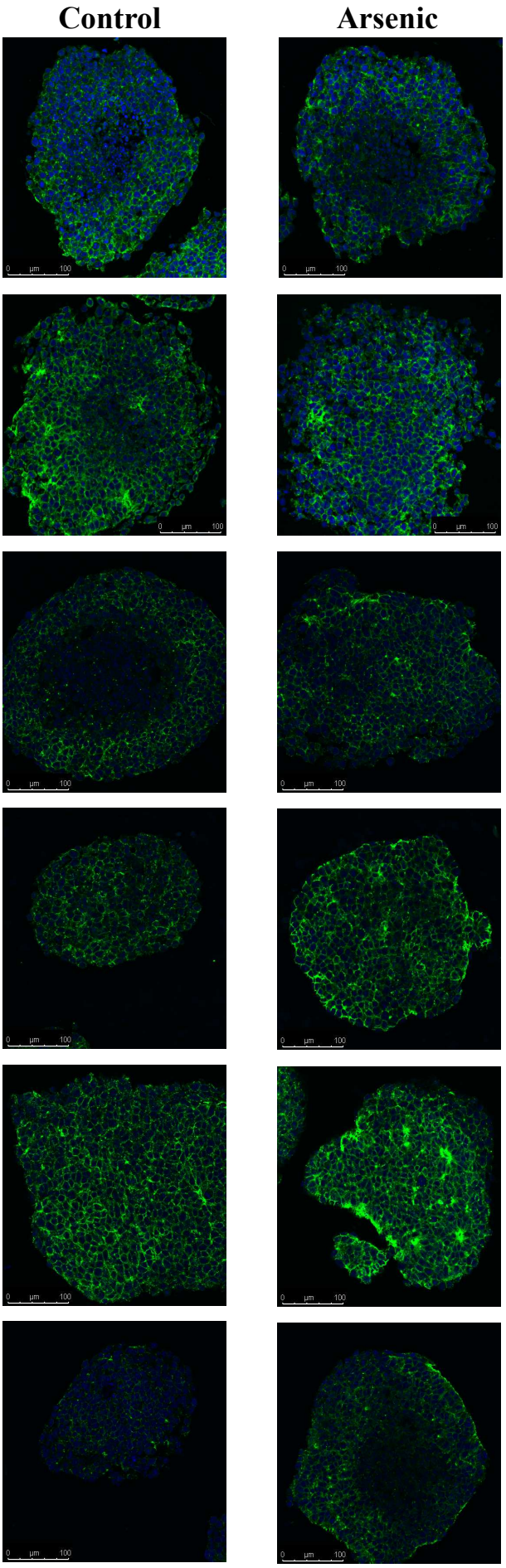
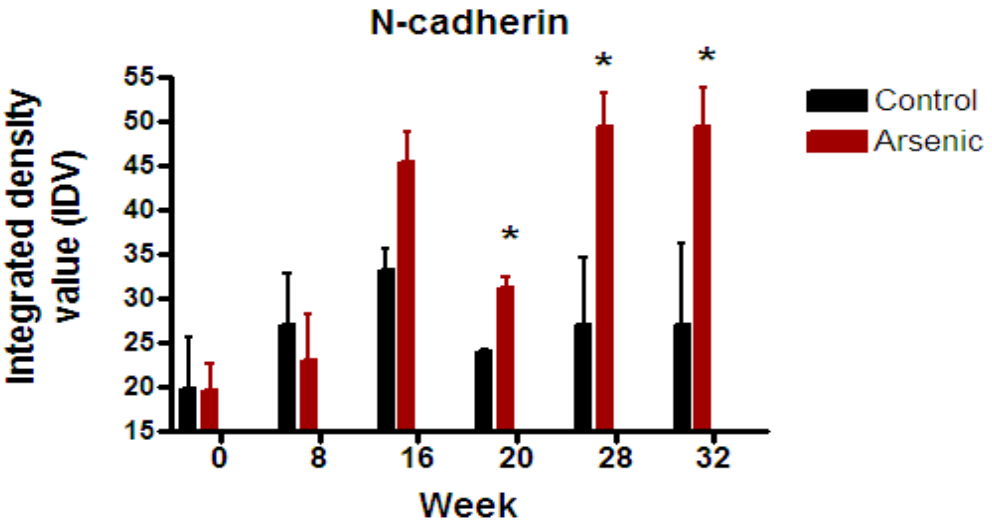

Sox2 Expression
Corrected density
8.0×10⁵
7.0×10⁵
6.0×10⁵
5.0×10⁵
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
*
*
Week 20
Week 24
Day 5

Fig. 6

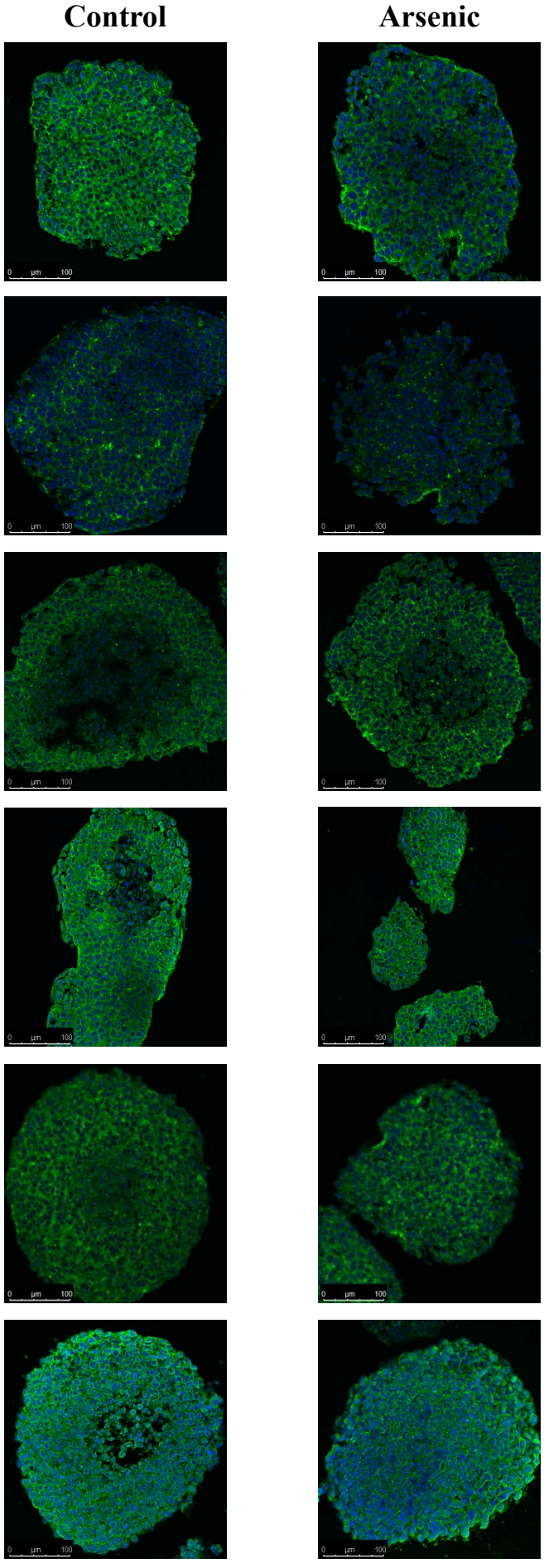
A.



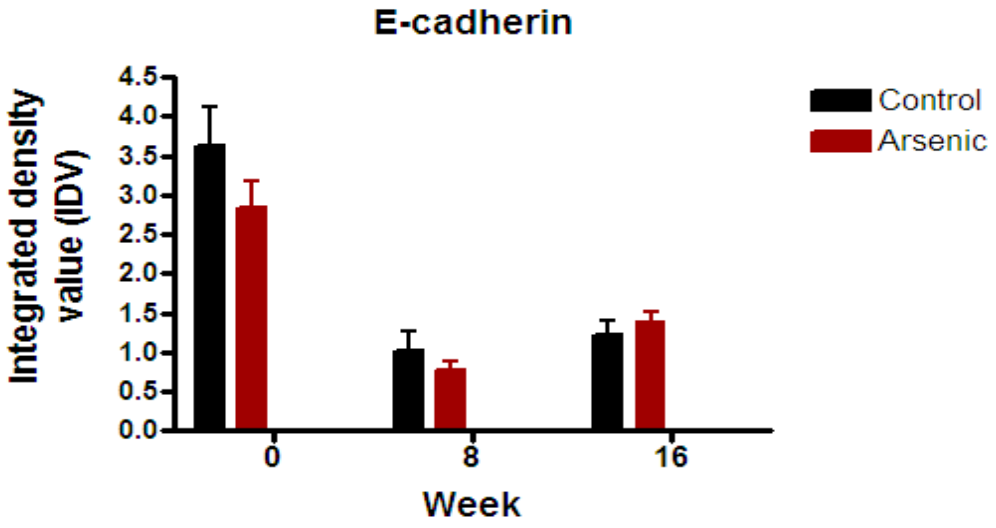
B.


N-cadherin
Integrated density value (IDV)
55
50
45
40
35
30
25
20
15
0
8
16
20
28
32
Week
*
*
*
Control
Arsenic

C.



D.


E-cadherin
Integrated density value (IDV)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
8
16
Week
Control
Arsenic

Chapter Three

Conclusion

Many studies have reported developmental deficits including decreased birthweight and lowered intelligence quotient (IQ) scores in children following chronic arsenic exposure during embryonic and fetal development (Rahman 2009, Wasserman 2014). Arsenic concentrations in drinking water are known to be substantially higher than the resulting blood concentrations following ingestion. However, arsenic will readily cross the blood-placenta-barrier, and concentrations in maternal blood are often comparable to levels found in cord blood (Concha 1998, Jin 2006). An epidemiological study of an Andean population in Argentina with concentrations of 200 μg/L arsenic in drinking water reported that median maternal blood arsenic concentrations were 11 μg/L, while median cord blood levels were 9 μg/L arsenic (Concha 1998). Therefore, the 7.5 μg/L arsenic concentration of the current study is a highly relevant concentration for the investigation of arsenic-induced developmental impairment.

While it is accepted that arsenic impacts development, there is much less understood about how arsenic causes these deficits at the molecular level. This study found that a chronic, *in vitro* 0.1 μM arsenic exposure maintains ESC pluripotency and impairs neuroblast differentiation. The expression of Sox2/Oct4 mRNA levels were first shown to be elevated at week 8 by 1.7-fold, while Sox2 protein expression began to be upregulated at week 16 of exposure by 1.4-fold and remained significantly elevated through the remaining exposure. Protein expression of N-cadherin beginning at week 20 by 1.3-fold also remained significantly increased through week 32 of exposure suggesting that an

EMT-like mechanism may be at least partially responsible for maintained pluripotency. Interestingly, these trends were exacerbated the longer the exposure continued. Further, decreased expression of the neurodevelopmental marker doublecortin was observed at week 12 and week 20 of exposure.

The current findings should be highly relevant to the field of public health as it is not uncommon for concentrations of arsenic greater than 200 μg/L to be found throughout the world in countries like Argentina and Bangladesh, as well as some areas of the United States (Concha 1998, Flanagan 2012, Nielson 2010). Though it has been shown previously that arsenic exposure can impair cellular differentiation and induce EMT, to our current knowledge this has not been shown in a chronic study at a level lower than the current drinking water standard of 10μg/L (10 ppb) (EPA 2001). This maintained pluripotency and EMT-like phenotype in embryonic stem cells may play a role in observed developmental deficits due to chronic *in -utero* arsenic exposure. For instance, the observed delayed and/or impaired neural differentiation of cells *in-vitro* may be indicative of delayed neural-fate determination in developing embryos, leading to learning deficits and reduced IQ scores.

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